

UNITED STATES PATENT AND TRADEMARK OFFICE

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Applicant : Kirkor Sirinyan
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AGENTS
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Docket No. : LeA 31923 C2

VIA EFS

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dr. Andreas Turberg, declares and states as follows:

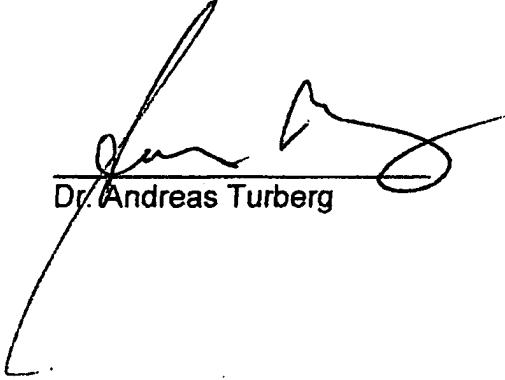
1. I received a Master of Science degree in Biology in 1986 from Heinrich-Heine-University, in Duesseldorf. Thereafter, I received a Doctorate in Natural Sciences from Heinrich-Heine-University University in Duesseldorf.
2. From 1992 to date, I have been employed by Bayer HealthCare AG, Monheim, Germany. My present position is Head of Laboratory Evaluation Arthropodicidal Drugs of the Department of Parasitology of Clinical Research and Development.
3. Under my direction and control, a study to evidence the synergistic effects of combinations of macrocyclic lactones and neonicotinoids of the present invention against arthropods was conducted. Two compositions of macrocyclic lactones and neonicotinoids were tested, one included

imidacloprid and moxidectin and one included eprinomectin and thiametoxam, as can be seen on the attached supporting documents (Attachment 1). The compositions were each tested by varying the concentration of each active and observing the efficacy of the composition against arthropods, in particular *Lucilia cuprina* larvae.

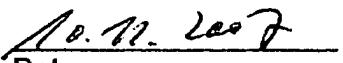
4. As detailed in Attachment 1, minced horse meat (1 cm³) was added to glass vials and supplemented with 500 µl of a compound solution of imidacloprid and ivermectin (concentration range max. 1000 to min. 0.01 ppm, specific concentrations are given in the respective experiment). 30 to 50 larvae were added and the vials were incubated for 48 hrs at 26°C ± 1.5°C and 60% ± 10 % relative humidity. The inhibition of larval development was monitored after 48 hours. Rating: IGR/mortality: 100 % efficacy = no larvae after 48 hrs, 0 % efficacy = normally developed larvae after 48 hrs.
5. As can be seen from Table 1 and Figure 1 there is a range of mixtures that show over-additive effects against arthropods in the *Lucilia* blowfly larval development assay over the respective compounds alone. While none of the single actives shows efficacy in the concentrations 0.1 and 0.4 ppm (imidacloprid) or 0.5, 0.7 and 1 ppm (moxidectin) mixtures of these concentrations resulted in full efficacy against the blowfly larvae. Table 2a and Table 2b compare the LD50 values (i.e., dose of the composition that causes death in 50% of the larvae) of synergized and non-synergized Imidacloprid and Moxidectin respectively. The LD50 value was calculated from the dose-response curves using excel-plug-in XL-fit.
6. The same experimental set-up was then used on a compound solution of eprinomectin and thiametoxam. As can be seen from Table 3 and Figure 2 there is a range of mixtures that show over-additive effects against arthropods in the *Lucilia* blowfly larval development assay over the

respective compounds alone. While none of the single actives shows efficacy in the concentrations 0.3, 0.7, 1 and 3 ppm (thiametoxam) or 0.03, 0.07 and 0.1 ppm (eprinomectin), mixtures of these concentrations resulted in full efficacy against the blowfly larvae. Table 4a and Table 4b compare the LD50 values of synergized and non-synergized thiametoxam and eprinomectin respectively. The LD50 value was calculated from the dose-response curves using excel-plug-in XL-fit.

7. As can be observed based on these results, the two chemical classes, macrocyclic lactones and the neonicotinoids act synergistically against arthropods.
8. The applicant further declares that all statements made herein are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.



Dr. Andreas Turberg



10.11.2007

Date

Evidence for synergistic effects in mixtures of macrocyclic lactones and neonicotinoids against arthropods demonstrated in the blowfly development assay (in-vitro assay with *Lucilia cuprina* larvae)

A matrix assay design was applied in the *Lucilia cuprina* larval bioassay to demonstrate the synergistic enhancement of efficacy of mixtures of macrocyclic lactones (e.g. avermectines/ milbemycins, esp. moxidectin/eprinomectin) and neonicotinoids (e.g. chloronicotinyls/ chlorothiazolyls, esp. imidacloprid/thiametoxam).

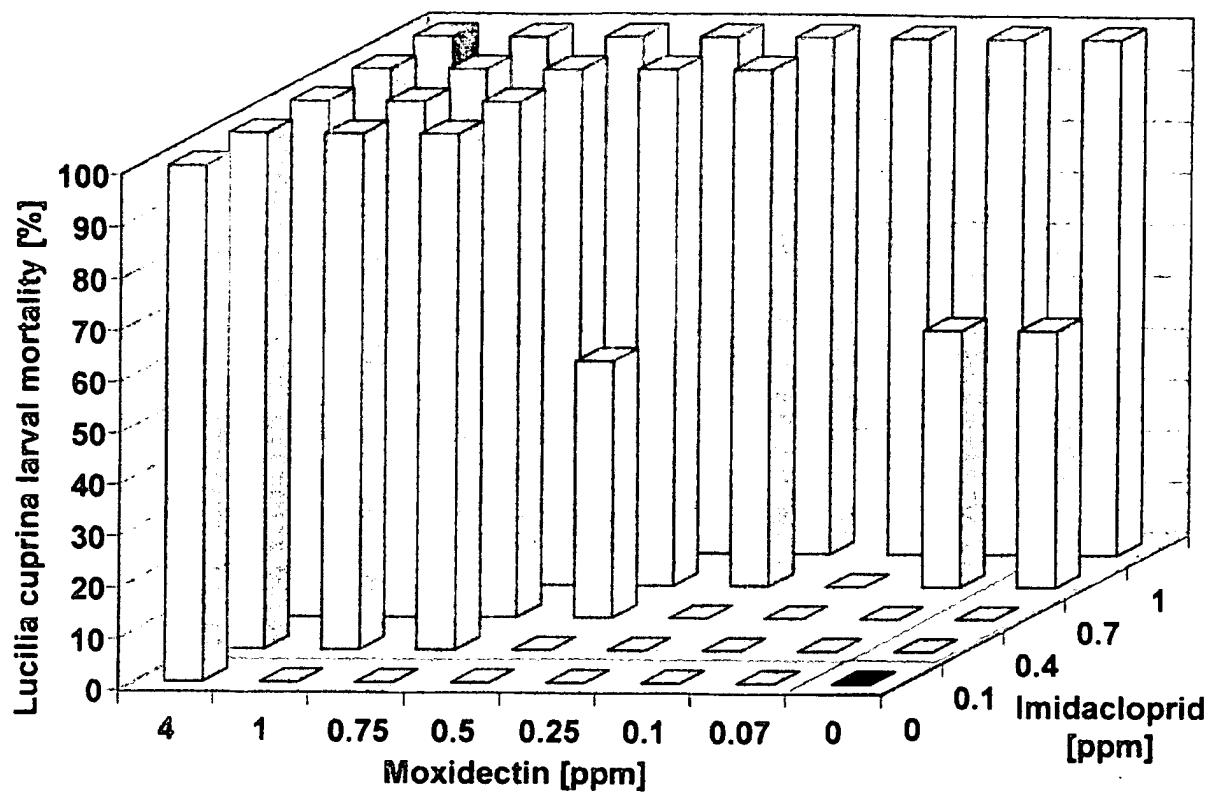
Test Method: Blowfly Development Assay - In vitro-assay with *Lucilia cuprina*: Arthropod: First to third larval instars (*Lucilia cuprina*), fully susceptible laboratory strain; Assay procedure: Minced horse meat (1 cm³) are added to glass vials and supplemented with 500 µl of a compound solution (concentration range max. 1000 to min. 0.01 ppm, specific concentrations are given in the respective experiment). 30 to 50 larvae are added and the vials are incubated for 48 hrs at 26°C ± 1,5°C and 60% ± 10 % relative humidity. Larval development is monitored after 48 hrs; Assay criteria: Inhibition of larval development (48 hrs); Rating: IGR/mortality: 100 % efficacy = no larvae after 48 hrs, 0 % efficacy = normally developed larvae after 48 hrs.

Table 1: Results from *Lucilia cuprina* larval development assay
(arithmetic means from 2 independent experiments performed in duplicates)

		Imidacloprid [ppm]								
		0	0.1	0.4	0.7	1	2.5	5	7.5	10
Moxidectin [ppm]	0	0	0	0	50	100	100	100	100	100
	0.07	0	0	0	50	100	100	100	100	100
	0.1	0	0	0	0	100	100	100	100	100
	0.25	0	0	0	100	100	100	100	100	100
	0.5	0	0	50	100	100	100	100	100	100
	0.75	0	100	100	100	100	100	100	100	100
	1	0	100	100	100	100	100	100	100	100
	4	100	100	100	100	100	100	100	100	100
	7	100	100	100	100	100	100	100	100	100
	10	100	100	100	100	100	100	100	100	100
	100	100	100	100	100	100	100	100	100	100

Shaded areas with bold figures are shown in Figure 1. Untreated control = white background; Light grey = compounds alone; Dark grey = mixture

Figure 1: Graphic representation of the relevant results from *Lucilia cuprina* larval development assay (arithmetic means from 2 independent experiments performed in duplicates)



Column color code	background	Group
■		Untreated control
□		Mixture, overlap of the lowest full efficacy doses
△		Moxidectin alone
▽		Mixture with lowest full efficacy dose of Moxidectin
▽		Imidacloprid alone
▽		Mixture with lowest full efficacy dose of Imidacloprid
▽		Mixture with no efficacy or no synergistic efficacy
▽		Mixture with synergistically enhanced efficacy

Table 2a: LD50 improvement for Imidacloprid by addition of Moxidectin

		ppm Moxidectin added				
		none	0.25	0.5	0.75	1
	Efficacy % of Moxidectin at ppm added:	na	0	0	0	0
Imidacloprid	LD50 [ppm]	0.7	0.57	0.4	0.03	0.03
	Improvement factor	na	1.2	1.8	23°	23°

Table 2b: LD50 improvement for Moxidectin by addition of Imidacloprid

		ppm Imidacloprid added			
		none	0.1	0.4	0.7
	Efficacy % of Imidacloprid at ppm added:	na	0	0	50
Moxidectin	LD50 [ppm]	2	0.62	0.45	0.2*
	Improvement factor	na	3.2°	4.4°	10°

* baseline corrected for 50% efficacy seen with Imidacloprid alone

° significant synergistic effects

As can be seen from Table 1 and Figure 1 there is a range of mixtures that show over-additive effects against arthropods in the *Lucilia* blowfly larval development assay over the respective compounds alone. While none of the single actives shows efficacy in the concentrations 0.1 and 0.4 ppm (imidacloprid) or 0.5, 0.7 and 1 ppm (moxidectin) mixtures of these concentrations resulted in full efficacy against the blowfly larvae. Table 2a and Table 2b compare the LD50 values of synergized and non-synergized Imidacloprid and Moxidectin respectively. The LD50 value was calculated from the dose-response curves using excel-plugin XL-fit.

The 0.7 ppm LD50 of Imidacloprid alone was enhanced to 0.03 ppm by addition of 0.75 or 1 ppm Moxidectin resulting in a 23-fold synergistic effect.

The 2 ppm LD50 of Moxidectin alone was enhanced to 0.62 ppm by the addition of 0.1 ppm Imidacloprid, to 0.45 ppm by addition of 0.4 ppm Imidacloprid and to 0.2 ppm by the addition of 0.7 ppm Imidacloprid respectively, resulting in a 3.2-fold, a 4.4-fold and a 10-fold synergistic effect.

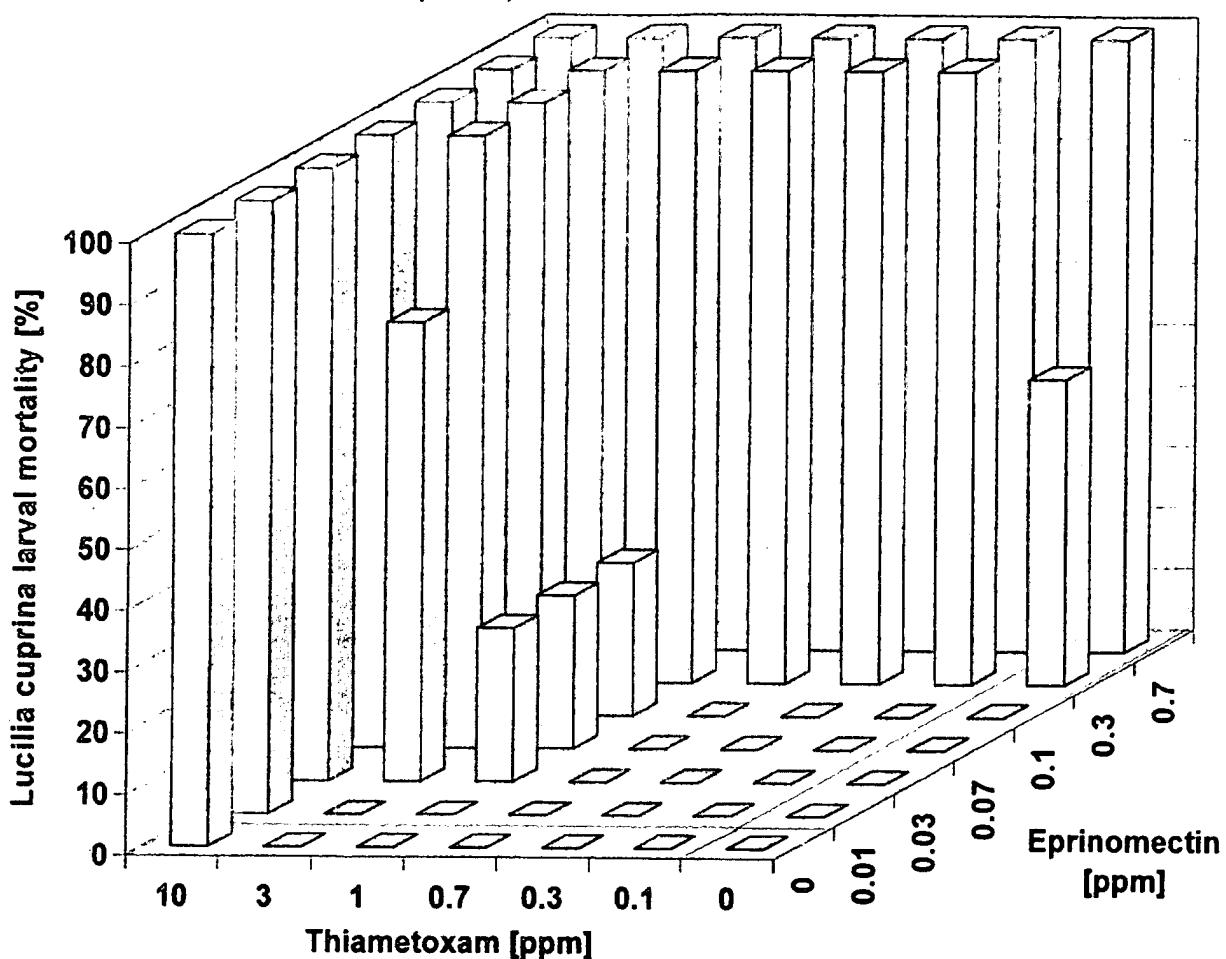
The same experimental set-up has been used with another pair of avermectin/neonicotinoide of different chemical structure: Thiametoxam and Eprinomectin.

Table 3: Results from *Lucilia cuprina* larval development assay
 (arithmetic means from 2 independent experiments performed in duplicates)

		Thiametoxam [ppm]							
		0	0.1	0.3	0.7	1	3	10	30
Eprinomectin [ppm]	0	0	0	0	0	0	100	100	
	0.01	0	0	0	0	0	0	100	100
	0.03	0	0	0	0	25	75	100	100
	0.07	0	0	0	0	25	100	100	100
	0.1	0	0	0	0	25	100	100	100
	0.3	50	100	100	100	100	100	100	100
	0.7	100	100	100	100	100	100	100	100
	1	100	100	100	100	100	100	100	100
	3	100	100	100	100	100	100	100	100

Shaded areas with bold figures are shown in Figure 2. Untreated control = white background; Light grey = compounds alone; Dark grey = mixture

Figure 2: Graphic representation of the relevant results from *Lucilia cuprina* larval development assay (arithmetic means from 2 independent experiments performed in duplicates)



Column color code	background	Group
---		Untreated control
---		Mixture, overlap of the lowest full efficacy doses
---		Thiametoxam alone
---		Mixture with lowest full efficacy dose of Thiametoxam
---		Eprinomectin alone
---		Mixture with lowest full efficacy dose of Eprinomectin
---		Mixture with no efficacy or no synergistic efficacy
---		Mixture with synergistically enhanced efficacy

Table 4a: LD50 improvement for Eprinomectin by addition of Thiametoxam

		ppm Thiametoxam added				
		none	0.3	0.7	1	3
	Efficacy % of Thiametoxam at ppm added:	na	0	0	0	0
Eprinomectin	LD50 [ppm]	0.3	0.18	0.18	0.09	0.02
	Improvement factor	na	1.7	1.7	3.3°	15°

Table 4b: LD50 improvement for Thiametoxam by addition of Eprinomectin

		ppm Eprinomectin added			
		none	0.03	0.07	0.1
	Efficacy % of Eprinomectin at ppm added:	na	0	0	0
Thiametoxam	LD50 [ppm]	4.8	1.7	1.4	1.4
	Improvement factor	na	2.8°	3.4°	3.4°

° significant synergistic effects

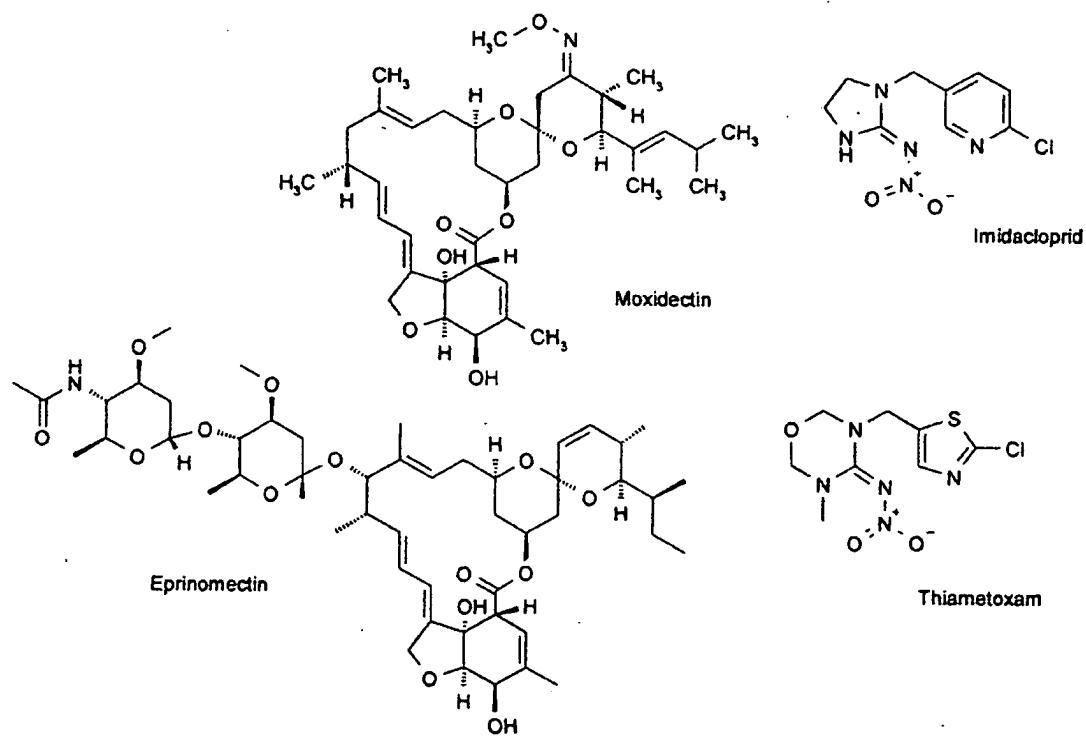
As can be seen from Table 3 and Figure 2 there is a range of mixtures that show over-additive effects against arthropods in the *Lucilia* blowfly larval development assay over the respective compounds alone. While none of the single actives shows efficacy in the concentrations 0.3, 0.7, 1 and 3 ppm (Thiametoxam) or 0.03, 0.07 and 0.1 ppm (Eprinomectin), mixtures of these concentrations resulted in full efficacy against the blowfly larvae. Table 4a and Table 4b compare the LD50 values of synergized and non-synergized thiametoxam and Eprinomectin respectively. The LD50 value was calculated from the dose-response curves using excel-plug-in XL-fit.

The 4.8 ppm LD50 of Thiametoxam alone was enhanced to 1.4 ppm by addition of 0.07 or 1 ppm Eprinomectin resulting in a 3.4-fold synergistic effect.. The same effect was observed when Thiametoxam was added to Eprinomectin. The Eprinomectin LD50 for *Lucilia* larvae of 0.3 ppm was improved to 0.09 ppm and 0.02 ppm by addition of 1 ppm and 3 ppm Thiametoxam resulting in a 3.3-fold and 15-fold synergistic effect, respectively.,

Conclusion

It has been demonstrated under the conditions of this controlled in-vitro experimental set-up that the two chemical classes, the macrocyclic lactones (avermectines/milbemycins) and the neonicotinoids (chloronicotinyls) act synergistically against arthropods (here: *Lucilia cuprina* larvae). This holds true for a variety of structural variants (synergistic efficacy of two exemplary pairs (Figure 3) has been shown here).

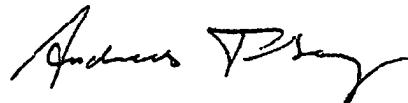
Figure 3: Structures of the macrocyclic lactone / neonicotinoide combinations used



The experiments have been performed the laboratory for Evaluation of Arthropodicidal Drugs in the Department of Parasitology of Clinical Research and Development (Bldg 6700) under the supervision of Dr. Andreas Turberg. The head of the laboratory and the executive staff have more than 13 years experience in testing of compounds against a variety of arthropods in different assay designs. The experiments have been carried out according to GSP standards. The experiments have been carried out according to SOP GSP-018-01 („Ablaufschema von Prüfaufträgen im Rahmen des biologischen Profilings_ In-vitro Ectoparasiten“).

Monheim, Sep. 1st, 2006 (part 1: moxidectin, imidacloprid)

Monheim, Nov. 24th, 2006 (part 2: eprinomectin, thiametoxam)



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Evaluation Arthropodicidal Drugs